Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Deletion of Talin1 in Myeloid Cells Facilitates Atherosclerosis in Mice

Huiping Shi,* Jianhua Song,* Liang Gao, Xindi Shan, Sumith R. Panicker, Longbiao Yao, Michael McDaniel, Meixiang Zhou, Samuel McGee, Hui Zhong, Courtney T. Griffin, Lijun Xia, Bojing Shao

BACKGROUND: Integrin-regulated monocyte recruitment and cellular responses of monocyte-derived macrophages are critical for the pathogenesis of atherosclerosis. In the canonical model, talin1 controls ligand binding to integrins, a prerequisite for integrins to mediate leukocyte recruitment and induce immune responses. However, the role of talin1 in the development of atherosclerosis has not been studied. Our study investigated how talin1 in myeloid cells regulates the progression of atherosclerosis.

METHODS: On an *Apoe*$^{-/-}$ background, myeloid talin1-deficient mice and the control mice were fed with a high-fat diet for 8 or 12 weeks to induce atherosclerosis. The atherosclerosis development in the aorta and monocyte recruitment into atherosclerotic lesions were analyzed.

RESULTS: Myeloid talin1 deletion facilitated the formation of atherosclerotic lesions and macrophage deposition in lesions. Talin1 deletion abolished integrin β2–mediated adhesion of monocytes but did not impair integrin α4β1–dependent cell adhesion in a flow adhesion assay. Strikingly, talin1 deletion did not prevent Mn^{2+}- or chemokine-induced activation of integrin α4β1 to the high-affinity state for ligands. In an in vivo competitive homing assay, monocyte infiltration into inflamed tissues was prohibited by antibodies to integrin α4β1 but was not affected by talin1 deletion or antibodies to integrin β2. Furthermore, quantitative polymerase chain reaction and ELISA (enzyme-linked immunosorbent assay) analysis showed that macrophages produced cytokines to promote inflammation and the proliferation of smooth muscle cells. Ligand binding to integrin β3 inhibited cytokine generation in macrophages, although talin1 deletion abolished the negative effects of integrin β3.

CONCLUSIONS: Integrin α4β1 controls monocyte recruitment during atherosclerosis. Talin1 is dispensable for integrin α4β1 activation to the high-affinity state and integrin α4β1–mediated monocyte recruitment. Yet, talin1 is required for integrin β3 to inhibit the production of inflammatory cytokines in macrophages. Thus, intact monocyte recruitment and elevated inflammatory responses cause enhanced atherosclerosis in talin1-deficient mice. Our study provides novel insights into the roles of myeloid talin1 and integrins in the progression of atherosclerosis.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis ■ cell adhesion ■ inflammation ■ integrins ■ myeloid cells

Atherosclerosis, a chronic inflammatory disease that manifests in large arteries, is the leading cause of cardiovascular disease. During atherosclerosis, monocyte recruitment from the circulation into the blood vessel wall plays a vital role in the initiation of inflammation.[1–3] Infiltrated monocytes differentiate into macrophages that ingest blood lipids and produce inflammatory cytokines, thereby promoting atherosclerosis.[4,5] Thus, prevention of monocyte entry into the blood vessel wall may lessen the formation of atherosclerotic lesions. However, the mechanism underlying monocyte recruitment during atherosclerosis is not well studied.

Integrins regulate leukocyte adhesion onto the blood vessel and extravasation into inflamed tissues.[6] Monocyte adhesion onto the inflamed vessel wall is mediated by integrins β2, including αLβ2 and αMβ2, and α4β1

Correspondence to: Bojing Shao, PhD, Laboratory of Vascular Inflammation and Thrombosis Research, Lindsley F. Kimball Research Institute, New York Blood Center, 310 E 67th St, New York City, NY 10065. Email bshao@nybc.org
*H. Shi and J. Song contributed equally.
Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/ATVBAHA.123.319677.
For Sources of Funding and Disclosures, see page 1811.

Arterioscler Thromb Vasc Biol is available at www.ahajournals.org/journal/atvb

Nonstandard Abbreviations and Acronyms

CXCL12	chemokine (C-X-C motif) ligand 12
DAPI	4′,6-diamidino-2-phenylindole
FITC	fluorescein isothiocyanate
HFD	high-fat diet
ICAM-1	intercellular adhesion molecule 1
IFN-γ	interferon-γ
IL	interleukin
LDV	Leu-1 N-2-[4-[[[(2 methylphenyl)amino] carbonyl]amino]phenyl]acetyl-, Lys-6 N
PE	phycoerythrin
RGD	arginylglycylaspartic acid (Arg-Gly Asp)
SMC	smooth muscle cell
TNF-α	tumor necrosis factor-α
VCAM-1	vascular cell-adhesion molecule 1

binding to endothelial ICAM-1 (intercellular adhesion molecule 1)[7–9] and VCAM-1 (vascular cell-adhesion molecule 1),[10,11] respectively. Reduction of VCAM-1 expression in mice partially impairs the formation of atherosclerotic lesions,[12] yet, deleting ICAM-1 presents inconsistent effects.[12–14] Furthermore, lacking the mechanistic picture of monocyte recruitment into atherosclerotic lesions confounds the findings. Therefore, more specific analysis of the contribution of integrins to monocyte recruitment in the context of atherosclerosis is warranted.

In the arterial wall, monocyte-derived macrophages uptake lipid particles, clear dead cells, and secrete cytokines or proteinases.[4,5] Integrins also play multifaceted roles in regulating macrophage functions during atherosclerosis. For example, integrin β3 negatively regulates the production of inflammatory cytokines by macrophages.[15] Smooth muscle cells (SMCs) in the blood vessel wall stabilize the atherosclerotic lesion.[16,17] Macrophages have been reported to regulate the proliferation of SMCs.[18] Whether integrins on macrophages play a role in the regulation of SMC proliferation during atherosclerosis remains elusive.

Talin1 is a ubiquitously expressed cytoskeletal protein.[19] Studies with patient samples show that talin1 expression in atherosclerotic plaque[20] and aortic media[21] is downregulated. Yet, it is unknown which cell types contribute to the reduced talin1 expression and whether this alteration plays a role in the initiation of atherosclerosis. Talin1 is well studied for its function in leukocyte trafficking. Talin1 binding to the cytoplasmic tail of integrin β subunits upregulates the integrin's affinity for ligands, thereby controlling leukocyte adhesion.[22] Talin1 is required for adhesion of leukocytes and platelets by integrins β2 and αIIbβ3, respectively.[23,24] Yet, the role of talin1 in leukocyte adhesion by integrin α4β1 is controversial.

Highlights

- Talin1 is dispensable for integrin α4β1 to adopt the high affinity for ligand binding and to mediate monocyte recruitment.
- Integrin α4β1 plays a dominant role in regulating monocyte recruitment into inflamed tissues.
- Integrin αvβ3 on macrophages inhibits the production of cytokines to promote inflammation and smooth muscle proliferation, and this process requires talin1.

Downregulation of talin1 by siRNA reduces integrin α4β1–mediated T-cell adhesion on VCAM-1[25] but does not impair adhesion of the U937 cell line.[26] Deletion of talin1 abrogates adhesion of mouse B cells on plates coated with a low density of VCAM-1 but does not interfere with cell adhesion on a high density of VCAM-1.[27] Whether talin1 is essential for upregulating the ligand-binding affinity of integrin α4β1 is not clear. In addition, how talin1 regulates integrin α4β1–mediated monocyte adhesion and recruitment has not been studied.

Here, we unexpectedly found that deletion of talin1 in myeloid cells, including monocytes, facilitates macrophage deposition in the lesions and the formation of atherosclerotic lesions in mice fed with a high-fat diet (HFD). Integrin β2–mediated monocyte adhesion required talin1, but integrin α4β1–mediated cell adhesion and integrin α4β1 activation to the high-affinity state were independent of talin1. Integrin α4β1 played a dominant role in monocyte recruitment into the inflamed tissues. In addition, talin1 was essential for integrin β3 to negatively regulate macrophages to produce cytokines and proteins that enhance inflammation and the proliferation of SMCs. Our findings provide novel insights into functions of integrins and macrophages in the development of atherosclerosis.

MATERIALS AND METHODS

Data Availability

The authors declare that all data, protocols, and analytic methods that support the findings of this study are available from the corresponding author upon reasonable request.

All data needed to evaluate the conclusions in the article are present in the article and the Supplemental Material.

Mice

All mouse experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation. *Apoe*$^{-/-}$ mice (C57BL/6J) were purchased from The Jackson Laboratory (No. 002052). Mice lacking talin1 in myeloid cells (LysMCre^{+};*Tln1*$^{f/f}$) were generated by breeding *Tln1*$^{f/f}$ mice[28]

with LysMCre+ mice[24] on the C57BL/6J background. These *Tln1*$^{f/f}$ mice were further bred into the *Apoe*$^{-/-}$ background for at least 5 generations to generate *Apoe*$^{-/-}$/LysMCre^{-};*Tln1*$^{f/f}$ (control) and *Apoe*$^{-/-}$/LysMCre^{+};*Tln1*$^{f/f}$ (Mye *Tln1*$^{-/-}$) mice. All mice were 6 to 18 weeks old at the time of study.

Reagents

EasySep mouse monocyte isolation kit was purchased from STEMCELL Technologies (Vancouver, Canada). RNeasy kit and Omniscript RT kit were purchased from Qiagen. SYBR Green Master Mix was purchased from Bio-Rad. PE (phycoerythrin) anti-mouse CD115 (monoclonal antibody [mAb]; clone AFS98), fluorescein isothiocyanate (FITC) anti-mouse Ly6C (mAb; clone HK1.4), FITC anti-mouse CD18 (mAb; clone M18/2), FITC anti-mouse CD49d (mAb; clone R1-2), FITC anti-mouse F4/80 (mAb; clone BM8), FITC rat IgG2a control (clone RTK2758), FITC rat IgG2b control (clone RTK4530), PE rat IgG2b control (clone RTK4530), PE rat IgG2a control (clone RTK2758), Alexa647 anti-mouse CD31 (mAb; clone 390), PE-anti-FITC (mAb; clone FIT-22), anti-mouse F4/80 (mAb; clone BM8), PE hamster anti-mouse CD61 (mAb; clone 2C9.G2), mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; mAb; clone FF26A/F9), and ELISA (enzyme-linked immunosorbent assay) kits for measuring mouse plasma TNF-α (tumor necrosis factor-α), IFN-γ (interferon-γ), IL (interleukin)-1b, IL-6, and IL-10 levels were purchased from BioLegend (San Diego, CA). Alexa 594 anti-FITC (mAb; clone 1F8-1E4) was from Jackson Immuno Research (West Grove, PA). Rat anti-mouse CD49d (mAb; clone phosphate-buffered saline [PBS]/2) was from Bio X Cell (Lebanon, NH).[29] Rat anti-mouse β2 integrin (mAb; clone GAME-46) was from BD Biosciences (San Jose, CA).[30] Rat anti-mouse macrophages (mAb; clone MOMA-2) were from Bio-Rad (Hercules, CA). Mouse anti-talin (mAb; clone 8d4) was from Sigma (St. Louis, MO). HRP (horseradish peroxidase)-conjugated goat anti-mouse IgG (polyclonal, polyclonal antibody) was from Thermo Fisher Scientific (Waltham, MA). Recombinant mouse P-selectin, recombinant mouse ICAM-1, recombinant mouse VCAM-1, recombinant mouse CXCL1 (chemokine (C-X-C motif) ligand 1), and recombinant mouse CXCL12 (chemokine [C-X-C motif] ligand 12) were purchased from R&D Systems, Inc (Minneapolis, MN). Recombinant mouse m-CSF (macrophage colony-stimulating factor) was purchased from BioLegend (San Diego, CA). The optical coherence tomography solution (Tissue-Tek) was from Sakura Finetek USA (Torrance, CA). Jurkat cells (clone E6-1) were from ATCC (Manassas, VA). CytoTrace Red CMTPX and Green CMFDA were from Cayman Chemical (Ann Arbor, MI). Leu-1 N-2-[4-[[[(2 methylphenyl)amino]carbonyl]amino] phenyl]acetyl-, Lys-6 N-FITC (FITC-Leu-1 N-2-[4-[[[(2 methylphenyl)amino]carbonyl]amino]phenyl]acetyl-, Lys-6 N [LDV]) and Bio1211 were purchased from Tocris (Minneapolis, MN). HFD was from Envigo (No. TD.88137, Indianapolis, IN). EnzyChrom Phospholipid Assay Kit was purchased from BioAssay Systems (No. EPLP-100, Hayward, CA). The standard laboratory diet for mice was PicoLab Rodent Diet 20, No. 5053, from Purina LabDiet (Richmond, IN). The major content of this regular diet is a crude protein (not less than 20%), crude fat (not less than 4.5%), crude fiber (not greater than 6%), and ash (a crude measurement of the total amounts of mineral, not greater than 7%).

HFD-Induced Mouse Atherosclerosis

Six-week-old male Mye *Tln1*$^{-/-}$ mice and the control mice on an *ApoE*$^{-/-}$ background were fed a Western diet (21% [wt/wt] fat; 0.3% cholesterol; research diet, No. D02031507) for 8 or 12 weeks. Mice were euthanized, and the circulatory system was flushed with PBS and then perfused with 4% paraformaldehyde. The heart and the aorta from the aortic root to the iliac bifurcation were removed and placed in 4% paraformaldehyde overnight. To analyze atherosclerotic lesion areas, the aorta was cut longitudinally to expose the intimal surface and stained with oil red O. Lesions in the aorta were quantified from the aortic arch, which was from the beginning of the ascending arch to 5 mm distal to the left subclavian artery to the iliac bifurcation. The lesion area (oil red O–positive area/total lumen area, %) was quantified with Image J software (NIH Image USA). The heart was further incubated with 20% sucrose-PBS, embedded in an OCT medium, and frozen. Lesions in the aortic root were analyzed following the recommendations of the American Heart Association.[31] Briefly, the hearts were sectioned perpendicular to the axis of the aorta from the inside of the heart toward the aortic arch. Once 3 valve leaflets of the aortic sinus were evident, consecutive sections at 10-μm thickness were collected until only 1 valve was observed in the aortic sinus. Four sections with recognizable 3 aortic valves starting from the first section with 3 leaflets together with the following 3 every-other sections (section No. 1, 3, 5, and 7) were used for analysis. These 4 cross sections that covered ≈70 μm of the aortic root were stained with nuclear dye (DAPI [4′,6-diamidino-2-phenylindole]), antibodies to macrophages (MOMA-2), and blood endothelium (CD31). Samples were imaged with a Nikon C^{2+} confocal microscope (Nikon Instruments Inc, NY) to acquire a series of *Z*-stack images at 1-μm intervals and were analyzed with NIS-element AR software (Nikon). The percentage of MOMA-2–positive lesion of total lesions and the pixels of MOMA-2–positive area were measured. The mean value of these 4 sections was used to represent the macrophage deposition of this group of samples from 1 mouse. Samples from a total of 8 mice were studied.

Preparation of Monocytes and Macrophages

Mouse monocytes were isolated from the bone marrow and peripheral blood of both male and female mice with EasySep mouse monocyte isolation kit as instructed by the manufacturer. The bone marrow cells were isolated by flushing femurs and tibias. BMDM (bone marrow–derived macrophages) were prepared as previously described.[32] Briefly, the bone marrow cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 15 ng/mL m-CSF at 37 °C in 5% CO_2. After culturing for 5 days, the medium was changed every day for another 5 days. Those cells were uniformly positive for mature macrophage markers F4/80 and Mac-1 (macrophage-1 antigen; integrin aMb2) by flow cytometry. For experiments, macrophage cells were depleted of both serum and m-CSF by overnight incubation.

Adhesion Assay Under Flow Conditions

The adhesion of monocytes and macrophages under flow was assessed on a parallel plate flow chamber. Briefly, a 35-mm tissue culture dish (Corning Glass Works, Corning, NY) was coated with P-selectin (0.2 μg/mL) and ICAM-1 (1.0 μg/mL)

with or without CXCL12 (2 μg/mL) or coated with VCAM-1 (0.2 μg/mL) with or without CXCL12 (2 μg/mL) in a solution of 10 mmol/L $NaHCO_3$ (pH=8.5) at 4 °C overnight. After blocking the chamber with 2% human serum albumin for 2 hours, monocytes or macrophages (1×10^6/mL) were perfused over the coated plate at 1 dyne/cm^2. After 5 minutes, adherent cells were imaged using a video microscope coupled to a digital analysis system on a Silicon Graphic workstation. In some experiments, cells were pretreated with antibodies to β2 integrin or antagonists to β1 integrin (Bio1211) before perfusion.

In Vivo Competitive Monocyte Adhesion and Transmigration

Purified monocytes (10^7) from control or Mye *Tln1*$^{-/-}$ mice were fluorescently labeled with 2-μM CellTracker Green CMFDA or CellTracker Orange CMRA dye, mixed, and retro-orbitally injected into TNF-α (1 μg)–challenged wild-type mice. Anti-CD31-Alexa 647 mAb was injected to label vessels. At 2 hours after injection, competitive adhesion and migration of injected, fluorescently labeled monocytes were visualized in the same venules (35–65 μm in diameter) of the cremaster muscle using spinning-disk confocal microscopy. Monocyte arrest and migration were visualized by time-lapse recording every 30 seconds, using *Z* stacks of 5-μm step with a total thickness of 30 μm. Time-lapse videos were recorded continuously for 30 minutes up to 2 hours. Adherent cells were defined as monocytes that did not move for at least 30 seconds. The spinning-disk confocal microscope is composed of a Nikon Eclipse microscope (E600FN) with a 20×/0.95 W XLUM plan F1 water immersion objective, which is coupled to a confocal light path (Solamere Technology Group) based on a modified Yokogawa CSU-X1 head (Yokogawa Electric Corporation) and is driven by the National Institutes of Health acquisition software Micromanager. The images were captured by Micromanager 1.4 and analyzed with ImageJ software (National Institutes of Health). In some experiments, antibodies to integrin β2 (clone GAME46) or integrin α4 (clone PBS/2) were injected intravenously at 30 minutes before cell injection.

Measurement of Integrin α4β1 Activation

Integrin α4β1 activation was measured by detecting the conformational change of its extracellular domain with LDV as described previously.[33] Briefly, Jurkat cells and monocytes were suspended in Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} (3×10^6/mL) and incubated with 1 μg/mL of FITC-LDV at 37 °C for 30 minutes. For the flow cytometry analysis, cells were further incubated with Ca^{2+} (1 mmol/L), Mn^{2+} (2 mmol/L), or CXCL12 (1 μg/mL) at 37 °C for 15 minutes followed by dilution with HBSS without Ca^{2+} and Mg^{2+} to 0.5×10^6/mL. Then diluted cells were fixed with 2% paraformaldehyde at room temperature for 10 minutes, washed, and incubated with PE-conjugated anti-FITC to detect LDV binding. For LDV binding in adherent cells, LDV-preincubated monocytes were diluted with HBSS with Ca^{2+} and Mg^{2+} to 0.5×10^6/mL followed by addition to coverslips coated with VCAM-1 (1 μg/mL) with or without CXCL12 (2 μg/mL). After incubation at 37 °C for 30 minutes, nonadherent cells were removed and adherent cells were fixed with 2% PFA. Then, adherent cells were incubated with Alexa549-anti-FITC, antibodies to monocytes (F4/80), and nuclear dye (DAPI), and imaged with confocal microscopy.

Western Blot

Monocytes, neutrophils, and CD4$^+$ T cells from LysMCre$^+$;*Tln1*$^{f/f}$ mice and the control mice were lysed with 1% Triton X-100, and the cell lysates were probed by Western blotting using mAbs to talin1 (8d4) or to GAPDH, followed by HRP-conjugated secondary antibodies. Of note, these 2 proteins were probed on separated membranes, as the molecular weight of talin1 (≈270 kD) and GAPDH (≈30 kD) differs too much.

Adhesion of Neutrophils Under Flow Conditions

The adhesion of purified neutrophils was assessed on a parallel plate flow chamber under flow conditions. Briefly, a 35-mm tissue culture dish (Corning Glass Works, Corning, NY) was coated with P-selectin (0.2 μg/mL) and ICAM-1 (1.0 μg/mL) with or without CXCL1 (2 μg/mL) in a solution of 10 mmol/L $NaHCO_3$ (pH=8.5) at 4 °C overnight. After blocking the chamber with 2% human serum albumin for 2 hours, neutrophils (1×10^6/mL) were perfused over the coated plate at 1 dyne/cm^2. After 5 minutes, adherent cells were imaged using a video microscope coupled to a digital analysis system on a Silicon Graphic workstation. In some experiments, cells were pretreated with antibodies to β2 integrin before experiments.

Counting Peripheral Blood Cells

Blood cell counts were performed as described previously.[34]

Flow Cytometry

Flow cytometry was performed as described previously.[32]

Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from cultured BMDM using the RNeasy Kit as instructed by the manufacturer. Reverse transcription was performed using the Omniscript room temperature Kit as instructed by the manufacturer. The derived cDNA was subject to real-time quantitative polymerase chain reaction analysis by mixing with primers and SYBR Green Master Mix. The primer sequences were as follows: Gapdh forward 5′-TCTCCACACCTATGGTGCAA-3′ and reverse 5′-CAAGAAACAGGGGAGCTGAG-3′; IFN-γ forward 5′-CGGCACAGTCATTGAAAGCCTA-3′ and reverse 5′-GTTGCTGATGGCCTGATTGTC-3′; TNF-α forward 5′-TGCCTATGTCTCAGCCTCTTC-3′ and reverse 5′-GGTCTGGGCCATAGAACTGA-3′; IL-10 forward 5′-AAGGCAGTGGAGCAGGTGAA-3′ and reverse 5′-CCAGCAGACTCAATACACAC-3′; MCP-1 (monocyte chemoattractant protein-1) forward 5′-TTCCTCCACCACCATGCAG-3′ and reverse 5′-CCAGCCGGCAACTGTGA-3′; MMP2 (matrix metalloproteinase) forward 5′-AACTACGATGATGACCGGAAGTG-3′ and reverse 5′-TGGCATGGCCGAACTCA-3′; MMP9 forward 5′-CTGGACAGCCAGACACTAAAG-3′ and reverse 5′-CTCGCGGCAAGTCTTCAGAG-3′; Pdgfβ forward 5′-GCAAGACGCGTACAGAGGTG-3′ and reverse 5′-GAAGTTGGCATTGGTGCGA-3′; VEGFA (vascular endothelial growth factor A) forward 5′-GGACGGGCCTCCGAAACCATG-3′ and reverse 5′-GACGGCAGTAGCTTCGCTGGT-3′; Arg-1 (Arginase-1) forward 5′-GTGAAGAACCCACGGTCTGT-3′ and reverse 5′-CTGGTTGTCAGGGGAGTGTT-3′. Real-time quantitative polymerase chain reaction data acquisition and analysis were performed using the Bio-Rad CFX manager 3.0 software. The δCt method was used for the comparison of relative

expression while the ΔΔCt method was used to compare the relative normalized expression.

Measurement of Plasma Cytokines

Mouse plasma TNF-α, IFN-γ, IL-1b, IL-6, and IL-10 levels were measured by ELISA as instructed by the manufacturer. Briefly, 100 µL of capture antibody diluted in coating buffer B was added to each well of a 96-well plate and incubated overnight at 4 °C. After washing on the second day, 200 µL of assay diluent A was added to each well for blocking nonspecific binding. Mouse plasma samples were diluted 5× using 1× assay diluent A and measured in duplicate. Afterward, the plate was washed and added with 100 µL per well of standards or plasma samples. Following a 2-hour incubation at room temperature, the plate was washed and 100 µL of diluted detection antibody solution was added to each well for another 1-hour incubation at room temperature. The binding was detected by adding avidin-HRP and freshly mixed TMB (3,3′5,5′-tetramethylbenzidine) substrate solution sequentially. After the reaction was stopped by adding 100 µL per well of stop solution, the absorbance was read at 450 nm using FLUOstar Omega microplate reader.

Measurement of Mouse Blood Phospholipids

Mouse plasma phospholipid levels were measured using a Phospholipid Assay Kit. Briefly, mix 20 µL of each plasma sample with 80 µL working reagent (assay buffer: phospholipid enzyme: enzyme mix: dye reagent=85:1:1:1). Prepare the standards using the phosphatidylcholine provided in the kit. Tap the plate to mix and incubate for 30 minutes at RT, followed by an optical density reading at 570 nm.

Statistical Analysis

Statistical analyses were performed with Prism 10. For comparisons between 2 groups, unpaired *t* test was used when the normality assumption was met using the Shapiro-Wilk test. *F* test was used to check for equal variances. In case of unequal variances, the unpaired *t* test with Welch correction was used. If the normality assumption was not met, Mann-Whitney *U* test was used to analyze the difference between 2 groups. For quantitative polymerase chain reaction studies, 1-sample *t* test was performed to check the significance of analyzed genes with the hypothetical value of 1, which was acquired by normalized housekeeping gene. A *P* value of <0.05 was considered significant.

RESULTS

Deletion of Talin1 in Myeloid Cells Enhances the Formation of Atherosclerotic Lesions and Macrophage Deposition in the Lesions

Talin1 is essential for integrin-mediated neutrophil adhesion onto endothelium and extravasation into inflamed tissues.[35] Thus, we examined whether deleting talin1 prevents the development of atherosclerosis by inhibiting monocyte entry into the arterial vessel wall. LysMCre mice that carry floxed talin1 (*Apoe*$^{-/-}$/LysMCre^{+};*Tln1*$^{f/f}$, Mye *Tln1*$^{-/-}$) were used as a mouse model that lacks talin1 in myeloid cells, including monocytes. *Apoe*$^{-/-}$/LysMCre^{-};*Tln1*$^{f/f}$ (control) mice were used as control mice. Specific deletion of talin1 in myeloid cells, such as neutrophils and monocytes but not T cells, was confirmed by Western blots (Figure S1A through S1D) and by inhibition of integrin β2–mediated neutrophil adhesion on ICAM-1 (Figure S1E). We next placed these mice on an HFD for 8 and 12 weeks to induce atherosclerosis. The HFD induced the formation of lipid lesions in the aortas of control mice, which was revealed by en face oil-red staining (Figure 1A and 1B). Surprisingly, Mye *Tln1*$^{-/-}$ mice formed larger lipid lesions in their aortas (Figure 1A and 1B). In addition to the increased lesion formation, Mye *Tln1*$^{-/-}$ mice also accumulated more macrophages in their aortic sinuses than control mice after 8 weeks of HFD (Figure 1C and 1D; Figure S2). Yet, deletion of myeloid talin1 did not alter the count of peripheral blood cells (Figure S3A and S3B) and lipid metabolism (Figure S3C through S3E), suggesting that other mechanisms are responsible for the increased lesion formation and macrophage accumulation. In addition, the deletion of myeloid talin1 did not cause significant cell apoptosis (Figure S4) or alter cell proliferation (Figure S5) in the aortas. Thus, talin1 is dispensable for monocyte recruitment into the inflamed vessel and plays an unexpected antiatherosclerotic role, which is in contrast with the prevailing concept suggesting talin1 is required for leukocyte recruitment and inflammation development.

Deletion of Talin1 Does Not Affect Monocyte Migration Into Extravascular Tissues

Monocytes in the circulation are the major source of macrophages in atherosclerotic lesions,[36] and monocytes enter atherosclerotic lesions mainly through postcapillary venules.[37] Thus, we examined the role of talin1 in monocyte trafficking into lesions with a mouse model of TNF-α–challenged cremaster muscle. Adhesion and transmigration of labeled monocytes in the venules of the cremaster muscle were analyzed. Two hours after the TNF-α challenge, a similar number of monocytes of both genotypes adhered onto the blood vessel wall and extravasated into tissues (Figure 2A and 2B). Monocytes from control and Mye *Tln1*$^{-/-}$ mice with or without feeding of an HFD showed the same expression levels of adhesion molecules, including β1 integrin (ie, CD49d), β2 integrin (ie, CD11a and CD11b), and CD44 (Figures S6 and S7). Thus, talin1 is not essential for monocyte recruitment into inflamed tissues, which is consistent with our findings showing that talin1 is not required for macrophage deposition into atherosclerotic lesions and lesion formation (Figure 1C through 1E).

Integrin β1 Controls Monocyte Recruitment Into the Inflamed Tissue

It is thought that integrins α4β1 and αLβ2 regulate monocyte recruitment into inflamed tissues.[36] Yet, the

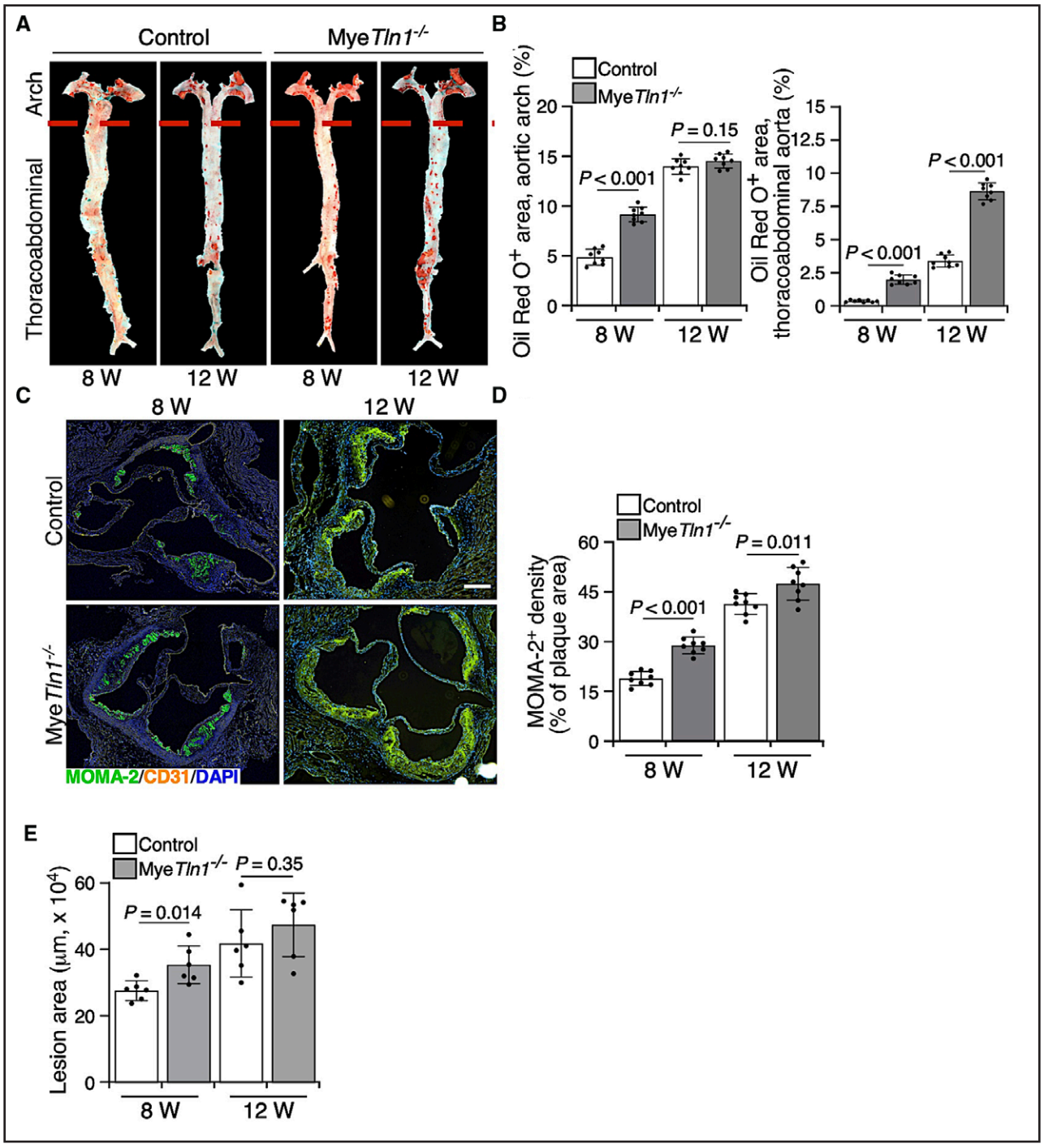

Figure 1. Myeloid talin1 deficiency enhances atherosclerosis and macrophage burden in atherosclerotic lesion.
A, Representative images of en face oil red O–stained aortas from control (*Apoe*$^{-/-}$/LysMCre^{-};*Tln1*$^{f/f}$) and Mye *Tln1*$^{-/-}$(*Apoe*$^{-/-}$/LysMCre^{+};*Tln1*$^{f/f}$) mice that were fed with a high-fat diet for 8 and 12 weeks. In each genotype, n=8. Red dashed line demarcated the aortic arch and thoracic aorta. **B**, Quantification of the area (%) of oil red O–positive lesion in aortic arch and thoracoabdominal aorta in aortas shown in **A**. **C**, Representative immunostaining images of transverse sections of aortic sinus from control and Mye *Tln1*$^{-/-}$ mice that were fed with a high-fat diet for 8 and 12 weeks. MOMA-2, an antibody recognizing mouse macrophages. CD31, a maker for endothelial cells on the blood vessel wall. **D**, Quantification of the MOMA-2–positive area (%) in transverse sections of aortic sinus shown in **C**. **E**, Quantification of the lesion area in transverse sections of aortic sinus. All data are acquired from 3 independent experiments, n=8 male mice per genotype, and results are expressed as mean±SD. Scale bar, 200 μm.

roles of these integrins in monocyte recruitment are still under debate. Thus, mAbs to integrin β2 or α4β1 were given to mice before infusion of fluorescently labeled monocytes and TNF-α challenge. Injection of blocking mAbs to integrin β2 did not alter the adhesion and migration of monocytes from the 2 genotypes

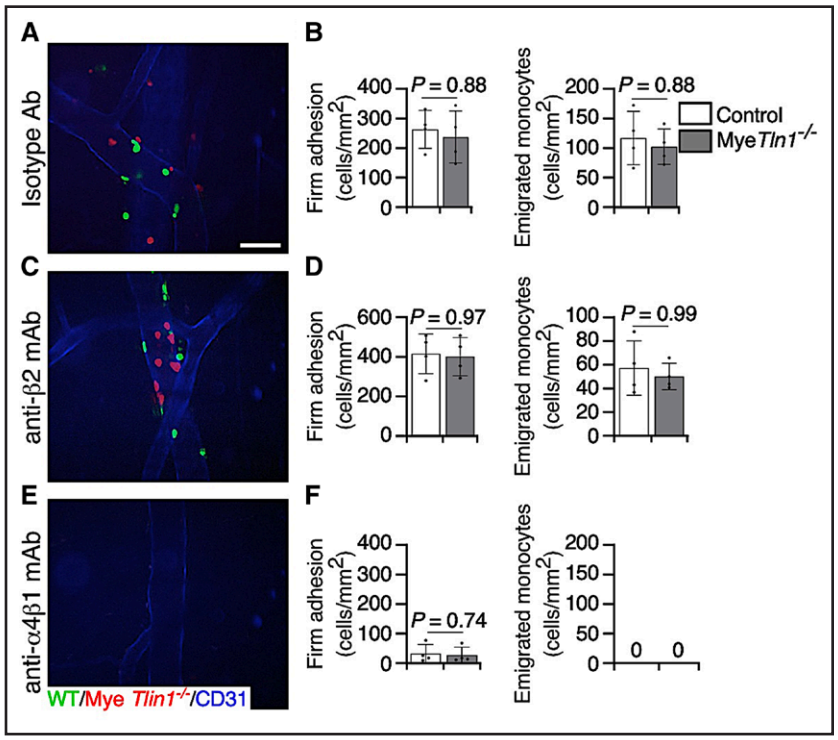

Figure 2. Integrin α4β1 dominantly contributes to monocyte adhesion on inflamed vessels and extravasation into tissues in vivo.
Adherent and extravasated monocytes from control (green) and Mye *Tln1*$^{-/-}$ (red) mice at 2 hours after intravenous injection into TNF-α (tumor necrosis factor-α)–challenged wild-type (WT) mice that were preinjected with (**A**) isotype Abs, and inhibitory Abs to (**C**) integrin β2 or (**E**) α4β1. Blood vessel endothelial cells were stained with anti-CD31 monoclonal antibody (mAb; blue). The images are representative of 4 independent experiments. Scale bar, 200 μm. **B**, **D**, and **F**, Quantification of adherent luminal and extravascular monocytes at 2 hours after injection was presented in the right of the images. The data represent mean±SD of 50 to 300 monocytes counted in 7 to 8 venules from each of 4 independent experiments. Each dot represents a mean from 1 mouse.

(Figure 2C and 2D). However, blocking mAbs to integrin α4β1 completely abolished adhesion and migration of the monocytes we assessed, regardless of genotype (Figure 2E and 2F). Furthermore, there was no difference in adherent and migrated cells between isotype- and Abs-to-integrin β2–treated mice. These results suggest that monocyte adhesion and extravasation in inflamed tissues are predominantly regulated by integrin α4β1 and that integrin β2 does not play a significant role in this process.

Talin1 Is Dispensable for Integrin α4β1–Mediated Adhesion of Monocytes and Macrophages

Integrin-mediated adhesion is a prerequisite for leukocyte extravasation into inflamed tissues. Thus, we examined the effect of talin1 deletion on adhesion mediated by integrins α4β1 and β2 underflow. Both monocytes and macrophages from control mice exhibited CXCL12-induced and β2 integrin–dependent adhesion on ICAM-1–coated plates (Figure 3A and 3B). However, monocytes and macrophages from Mye *Tln1*$^{-/-}$ mice did not adhere to immobilized ICAM-1 (Figure 3A and 3B), although these cells expressed normal levels of adhesion molecules, including selectin ligands and integrins (Figures S6 and S7). These results are consistent with previous studies in which talin1 deletion prohibited β2 integrin–dependent neutrophil adhesion.[24] Control monocytes and macrophages also showed integrin α4β1–mediated adhesion on immobilized VCAM-1 (Figure 3C and 3D). Surprisingly, talin1-deficient monocytes and macrophages exhibited a comparable adhesion on VCAM-1 (Figure 3C and 3D). These results suggest that talin1 is not required for integrin α4β1–mediated adhesion of monocytes and macrophages.

Integrin α4β1 Activation Is Intact in Talin1-Deficient Monocytes

Upregulation of ligand-binding affinity is critical for integrins to arrest leukocytes.[35] Thus, we tested whether talin1 is required for integrin α4β1 activation to its high-affinity state for ligands. LDV is a peptide that specifically recognizes human integrin α4β1 with a high ligand-binding affinity.[38] Mn^{2+}, which activates integrins allosterically,[39] increased LDV binding to the human Jurkat cell line (Figure 3E). Interestingly, LDV binding to monocytes from control mice was also increased by Mn^{2+} or CXCL12 (Figure 3F and 3G), suggesting that LDV also specifically recognizes activated mouse integrin α4β1. Compared with control monocytes, talin1-deficient monocytes exhibited a similar LDV binding in response to Mn^{2+} and CXCL12 (Figure 3F and 3G). These unexpected findings indicate that activation of integrin α4β1 to its high-affinity state is independent of talin1.

Next, we further examined the activation of integrin α4β1 on adherent monocytes. Monocytes were added to polylysine- or VCAM-1–coated plates, and LDV binding was assessed using fluorescent microscopy. LDV binding was not detected on poly lysine-captured monocytes, indicating that integrin α4β1 on these cells was not activated (Figure 3H). In contrast, LDV bound both control and talin1-deficient monocytes that adhered to

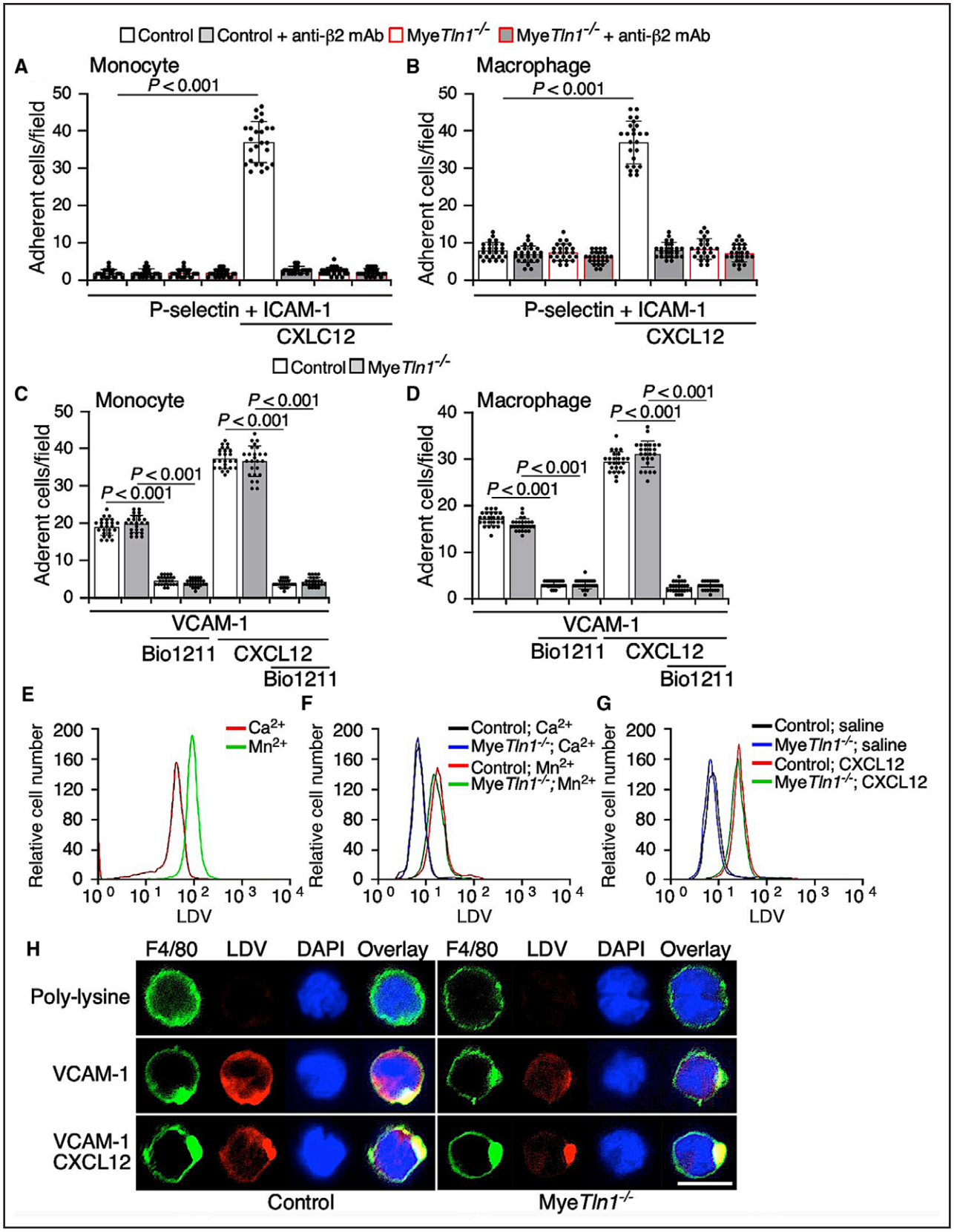

Figure 3. Integrin β1–mediated adhesion and integrin α4β1 activation are intact in talin1-deficient monocytes.
Adhesion of (**A**) monocytes and (**B**) macrophages of the indicated genotype on plates coated with P-selectin, ICAM-1 (intercellular adhesion molecule 1) in the presence or absence of CXCL12 (chemokine [C-X-C motif] ligand 12) at 1 dyne/cm². In some experiments, cells were pretreated with anti-integrin β2 antibodies before perfusing over the plates. Adhesion of (**C**) monocytes and (**D**) macrophages of (*Continued*)

VCAM-1 (Figure 3H). In the presence of CXCL12, LDV was clustered on adherent cells on VCAM-1 (Figure 3H). Thus, integrin α4β1 on adherent monocytes is activated and further forms clusters upon chemokine stimulation independently of talin1. These results are consistent with our in vivo studies that show integrin α4β1 is essential for monocyte recruitment into inflamed tissues, whereas talin1 is dispensable for this process (Figure 2).

Deletion of Myeloid Talin1 Enhances Inflammation in Mice

Lipidemia causes inflammatory responses that contribute to the progression of atherosclerosis. Thus, we measured the plasma levels of inflammatory cytokines in mice. Feeding the control mice with the HFD increased the plasma levels of inflammatory cytokines, including TNF-α, IL-1b, IL-6, IFN-γ, and IL-10 (Figure 4A through 4E). The HFD increased the levels of cytokines in Mye *Tln1*$^{-/-}$ mice, as well (Figure 4A through 4E). Yet, compared with control mice, Mye *Tln1*$^{-/-}$ mice had higher levels of cytokines before feeding with the HFD and at 8 weeks after the HFD (Figure 4A through 4E). The signals of TNF-α and IL-6 in the lesion were stronger in Mye *Tln1*$^{-/-}$ mice (Figure 4F and 4G; Figure S9), suggesting that deleting myeloid talin1 also increased inflammation in the atherosclerotic lesion.

Integrin β3 negatively regulates inflammatory cytokine production in macrophages,[15] and talin1 is required for integrin β3 to bind its ligands with high affinity.[40,41] Thus, we cultured macrophages and analyzed the expression of genes for inflammatory cytokines. Compared with macrophages from control mice, the gene expression of IFN-γ, TNF-α, and IL10 in macrophages from Mye *Tln1*$^{-/-}$ mice was increased (Figure 4H). In addition, pretreatment of macrophages from control mice with RGD (arginylglycylaspartic acid [Arg-Gly Asp]) peptide, which blocks ligand binding to integrin β3, increased the expression of genes for IFN-γ and TNF-α (Figure 4I). Thus, talin1 deficiency blocks the negative effects of integrin β3 on the production of inflammatory cytokines by macrophages, which may enhance inflammation in atherosclerosis.

Deletion of Myeloid Talin1 Increases SMCs in Atherosclerotic Lesions

SMCs are involved in the progression of atherosclerosis.[16,17] In HFD-fed mice, there were more SMCs in the aortas of Mye *Tln1*$^{-/-}$ mice (Figure 5A through 5C; Figure S10). Thus, we tested the role of talin1 in regulating the secretion of effector proteins for the proliferation of SMCs by macrophages. The gene expression of MMP2, MMP9, PDGFβ1 (platelet-derived growth factor β1), VEGFa, and Arg-1, which promote the proliferation of SMCs, was examined. Deleting talin1 increased the expression of MMP2, MMP9, and PDGFβ1 in macrophages (Figure 5D). As integrin β3–mediated signals may inhibit macrophages-producing effector proteins for the proliferation of SMCs,[18] we further tested the role of integrin β3 on macrophages in SMC proliferation. Blocking ligand binding to integrin β3 with RGD peptide increased the expression of MMP2, MMP9, and PDGFβ1, in control macrophages (Figure 5E). In addition, RGD peptide did not have effects on talin1-deficient macrophages (Figure 5F). Therefore, integrin β3 on macrophages may inhibit the proliferation of SMCs. Talin1 deficiency prohibits the negative regulation of integrin β3, which may increase SMCs in atherosclerotic aortas.

DISCUSSION

Monocyte recruitment and functions of monocyte-derived macrophages play critical roles in the progression of atherosclerosis.[36,42,43] However, the role of integrins in these cellular events is only partially understood. Here, our studies document the predominant role of integrin α4β1 in monocyte recruitment during atherosclerosis. In contrast to integrin β2, integrin α4β1 activation and monocyte adhesion by integrin α4β1 are independent of talin1. Our studies also show that integrin β3 negatively regulates macrophages to produce inflammatory cytokines and effector proteins, which could promote the proliferation of SMCs. These functions of integrin β3 require talin1. Of note, male mice with matched age and body weight were used for in vivo studies of the atherosclerosis development. Female mice were not used for in vivo studies as they are smaller in body weight, in general, and their sexual hormones may affect the study. Importantly, sex is not a factor associated with the functions of leukocyte integrins, which are the mechanistic focus of our study. Thus, this limitation would not cause a significant bias in interpreting data and drawing conclusions in our study.

Monocyte recruitment from the circulation into the intima of an inflamed vessel is essential for the development of atherosclerotic plaques.[6] It is thought that

Figure 3 Continued. the indicated genotype on plates coated with VCAM-1 (vascular cell-adhesion molecule 1) in the presence or absence of CXCL12 at 1 dyne/cm^2. In some experiments, cells were pretreated with Bio1211, an antagonist to integrin β1 before adhesion. Flow cytometry measurement of Leu-1 N-2-[4-[[[(2 methylphenyl)amino]carbonyl]amino]phenyl]acetyl-, Lys-6 N (LDV) binding to (**E**) Jurkat cells and (**F**) monocytes of the indicated genotype treated with Ca^{2+} or Mn^{2+}/EGTA. **G**, Flow cytometry measurement of LDV binding to monocytes of the indicated genotype treated with the vehicle control or CXCL12. **H**, Representative images of LDV-stained monocytes adhered on plates coated with poly lysine and VCAM-1 with or without CXCL12. All the results are the representative from 5 independent experiments, and the data represent mean±SD. Scale bar, 10 μm. DAPI indicates 4′,6-diamidino-2-phenylindole.

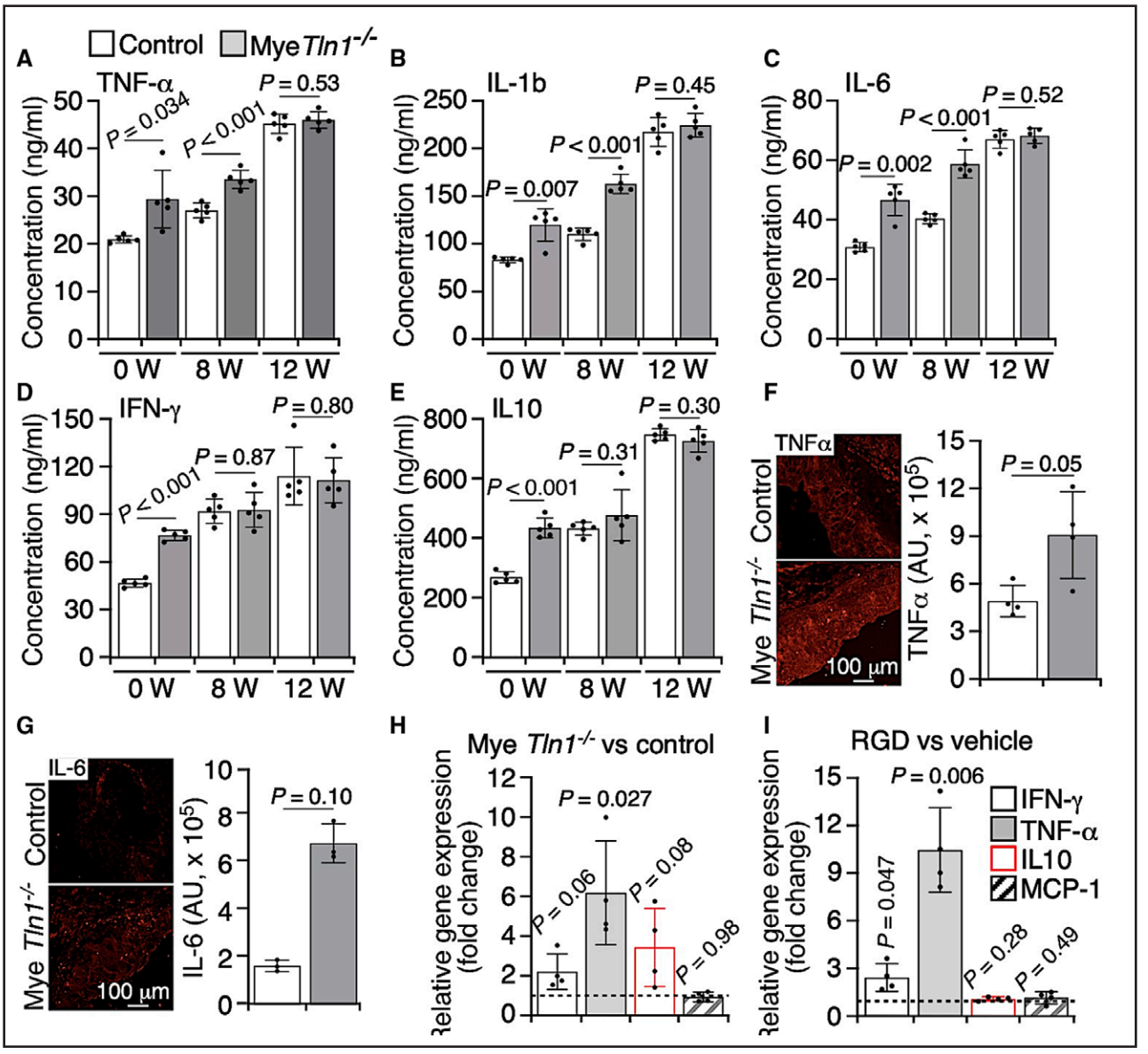

Figure 4. Myeloid talin1 deficiency increases inflammation at the early phase of atherosclerosis.
Measurement of the plasma levels of (**A**) TNF-α (tumor necrosis factor-α), (**B**) IL (interleukin)-1b, (**C**) IL-6, (**D**) IFN-γ (interferon-γ), and (**E**) IL-10 in control and Mye *Tln1*$^{-/-}$ mice at 0, 8, and 12 weeks after feeding with the high-fat diet. Plasma samples at each time point were from 8 mice of each genotype. Immunofluorescent staining of the transverse sections of aortic sinus from control mice and Mye *Tln1*$^{-/-}$ mice after feeding with a high-fat diet for 12 weeks with antibodies to (**F**) TNF-α and (**G**) IL-6 and quantification of the signals. **H**, Quantitative analysis of gene expression in macrophages from mice of the indicated genotypes. **I**, Quantitative analysis of gene expression in control macrophages treated with the vehicle control or RGD peptide, an antagonist to integrin β3. The dotted line represents the mean expression of the control gene, GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The results are from at least 4 independent experiments, and the data represent mean±SD.

monocyte recruitment follows the canonical cascade of leukocyte trafficking, which depends on adhesion molecules: selectins and integrins.[6] Of note, the role of adhesion molecules with respect to monocyte trafficking varies in differing inflammatory milieus. For example, monocyte recruitment into the thioglycollate-challenged peritoneal cavity depends on L-selectin,[44] whereas trafficking of monocytes to foci of *Listeria monocytogenes* infection in the liver is mediated by integrin β2 and CD44.[45] These discrepancies in monocyte recruitment may be attributed to varied surface receptors on heterogenous monocytes.[46] Studies show that the atherosclerotic vessel expresses ligands for both integrins β1and β2: VCAM-1 and ICAM-1.[47] Integrins α4β1 and αLβ2 support the adhesion of blood monocytes. However, the role of these 2 integrins in monocyte recruitment into atherosclerotic lesions is not clear. Our new data demonstrate that talin1 deletion abolishes integrin αLβ2–mediated monocyte adhesion but does not impair monocyte adhesion via integrin α4β1 (Figures 2 and 3). Furthermore, mAbs to integrin

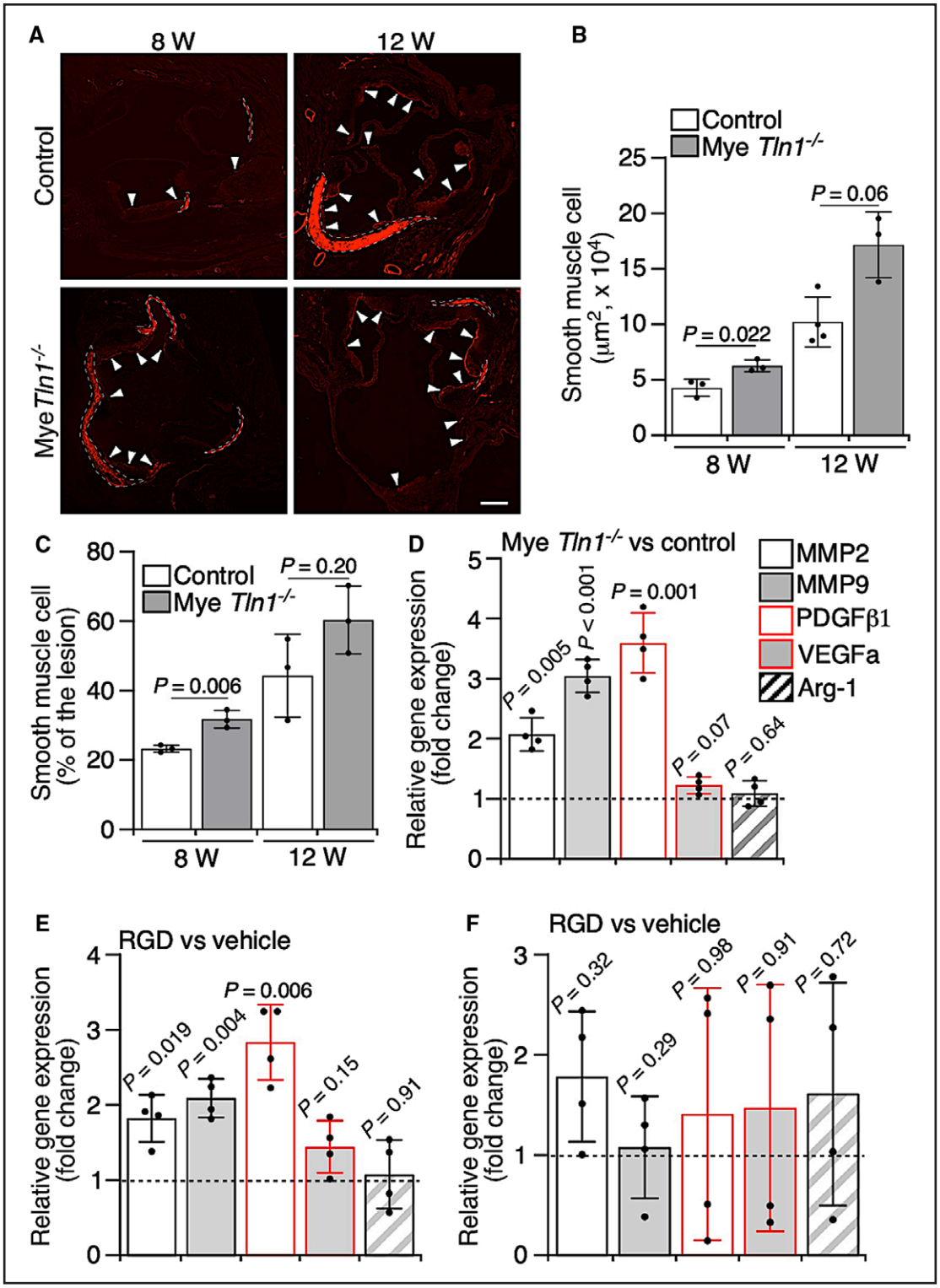

Figure 5. Myeloid talin1 deficiency increases smooth muscle cells (SMCs) in atherosclerotic aortas.
A, Representative images of transverse sections of aortic sinus from control and Mye *Tln1*$^{-/-}$ mice that were fed with a high-fat diet for 8 and 12 weeks. The cryosections were stained with antibodies to α-SMA (smooth muscle actin). Medial SMCs were demarcated with white dotted line, and SMCs in lesions were indicated with white arrowheads. In each genotype, n=8. Quantification of (**B**) the size of SMC-positive area and (**C**) the percentage of SMC-positive area in the lesions in transverse sections of aortic sinus. **D**, Quantitative analysis of gene expression in macrophages that were derived from monocytes of the indicated genotypes. **E**, Quantitative analysis of gene expression in control macrophages treated with the vehicle control or RGD peptide, an antagonist to integrin β3. **F**, Quantitative analysis of gene expression in myeloid talin1-deficient macrophages treated with the vehicle control or RGD peptide. The dotted line represents the mean expression of the control gene, GAPDH (glyceraldehyde-3-phosphate dehydrogenase). All the results are the representative from 4 independent experiments, and the data represent mean±SD. Scale bar, 200 μm. MMP indicates matrix metalloproteinase; PDGFβ1, platelet-derived growth factor β1; and VEGFA, vascular endothelial growth factor A.

α4β1, but not to αLβ2, block monocyte infiltration into inflamed tissues (Figure 2), and we found that deletion of talin1 in myeloid cells does not impair monocyte deposition in atherosclerotic lesions (Figure 1C). Thus, our studies indicate that integrin α4β1 plays a dominant role in monocyte recruitment into atherosclerotic lesions. Our results also agree with previous findings that downregulation of VCAM-1 partially reduces the formation of atherosclerotic lesions.[12] Therefore, direct blockade of integrin α4β1 may efficiently prevent the development of atherosclerotic lesions.

Talin1 deletion in myeloid cells surprisingly increases the formation of atherosclerotic lesions and macrophage deposition. Although intact adhesion by integrin α4β1 is essential and sufficient for recruitment of talin1-deficient monocytes, it should not increase monocyte recruitment and subsequent lesion formation. Instead of transmigration into the inflamed tissue, it has been noticed that neutrophils return from tissues to the blood circulation.[48] Under experimental conditions, monocytes can emigrate from atherosclerotic plaques into the circulation, which leads to the regression of atherosclerotic lesions.[49,50] Talin1 deletion may reduce monocyte emigration by inhibiting the function of some integrins, such as integrin β2, which would cause a subsequent increase of monocyte deposition and enlargement of lesions. In addition, the increased stabilization of atherosclerotic plaques in mice lacking myeloid talin1 may contribute to the formation of larger lesions.[1]

Talin1 binding to the integrin β tail upregulates the affinity of integrins for their ligands, thereby playing an essential role in integrin-mediated leukocyte adhesion.[22,51] Numerous studies have documented that talin1 is required for integrins β2 and β3 to firmly arrest leukocytes and platelets, respectively.[24,28] However, the role of talin1 in integrin α4β1–mediated leukocyte adhesion is controversial.[25,27,52] In addition, it is unknown whether talin1 is required for integrin α4β1 activation to the high-affinity state. In striking contrast to integrins β2 and β3, we found that talin1 is required for neither activation of integrin α4β1 to the high-affinity state, nor monocyte adhesion on VCAM-1 by integrin α4β1 (Figure 3). As integrin α4β1 is able to mediate leukocyte rolling on VCAM-1,[53] it is still not clear whether monocyte adhesion on VCAM-1 requires activation of integrin α4β1. In the future, it would be interesting to explore the mechanism by which integrin α4β1 is activated to the high-affinity state.

Infiltrated monocytes differentiate into macrophages, which produce cytokines and effector proteins further regulating the progression of atherosclerosis. Integrin β3 regulates many functional aspects of macrophages during atherosclerosis. For example, integrin β3 negatively regulates macrophages to produce inflammatory cytokines, which limits atherosclerosis.[15] Talin1 deletion or blockade of ligand binding to integrin β3 enhances the production of inflammatory cytokines (Figure 4), suggesting talin1 is essential for the negative regulatory role of β3. Studies of platelets document that talin1 binding to the β3 tail is required for activation of integrin αIIbβ3 and the subsequent integrin signaling upon ligand binding.[40,41] Thus, talin1 may be required for integrin β3 on macrophages to adopt the high affinity for ligands and the integrin β3 signaling as well.

SMCs in the neointima play a critical role in the progression of atherosclerosis. SMCs secrete matrix proteins to stabilize atherosclerotic lesions.[16,17] SMCs in the blood vessel wall positively correlate with the growth of atherosclerotic lesions.[54,55] The conditioned medium from integrin β3–deficient macrophages enhances the proliferation of SMCs,[18] suggesting integrin β3 mediates the production of effectors that negatively regulate SMC proliferation. Our studies show that integrin β3 inhibits the expression of MMP2/9 and PDGFβ1, leading to inhibition of SMC proliferation. Talin1 is required for the negative role of integrin β3, therefore lacking myeloid talin1 increases SMCs in atherosclerotic aortas, which may stabilize the lesion. Interestingly, studies with samples from patients with atherosclerosis show that talin1 is downregulated in unstable carotid plaques compared with stable plaques.[56] These findings suggest that reduced talin1 levels in plaques may cause the disintegration of the arterial wall in atherosclerosis. Thus, talin1 in differing cell types and stages of atherosclerosis may have varied pathological roles.

Our studies indicate that integrin α4β1 is more important than integrin αLβ2 for monocyte recruitment to atherosclerotic lesions. Furthermore, talin1 is dispensable for activation of integrin α4β1 and monocyte adhesion on VCAM-1. Talin1 deletion blocks the negative regulatory roles of macrophage integrin β3 in inflammation formation and SMC proliferation, which explains the increased atherosclerosis seen in Mye *Tln1*$^{-/-}$ mice. In summary, our studies clarify the roles of integrins α4β1 and β3 and their regulation by talin1 in the pathogenesis of atherosclerosis. Our findings could provide insights into the development of novel therapeutics for atherosclerosis.

ARTICLE INFORMATION

Received June 1, 2023; accepted April 23, 2024.

Affiliations

Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation (H.S., J.S., L.G., X.S., S.R.P., L.Y., M.M., M.Z., S.M., C.T.G., L.X., B.S.). Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center (H.S., L.X.). Lindsley F. Kimball Research Institute, New York Blood Center (H.Z., B.S.).

Acknowledgments

The authors thank Dr Rodger P. McEver in the Oklahoma Medical Research Foundation for providing the transgenic mice and critical scientific suggestions in project design. The authors also thank the University of Leicester, United Kingdom, for allowing us to use talin1 floxed mice (*Tln1*$^{f/f}$), a tremendously important mouse mode in our study. H. Shi, J. Song, L. Gao, X. Shan, S.R. Panicker, L. Yao, M. McDaniel, M. Zhou, S. McGee, H. Zhong, and B. Shao performed experiments. C.T. Griffin provided key reagents and interpreted data. L. Xia and B. Shao conceived

the study and interpreted data. H. Shi, J. Song, C.T. Griffin, L. Xia, and B. Shao wrote the article, with input from all authors.

Sources of Funding

The study is supported by grants from the National Institutes of Health (HL149860, HL153728, HL128390, HD083418, and GM114731 for L. Xia, R35HL144605 for C.T. Griffin, and AHA18CDA34120009 for B. Shao).

Disclosures

None.

Supplemental Material

Figures S1–S10
Major Resources Table

REFERENCES

1. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell*. 2011;145:341–355. doi: 10.1016/j.cell.2011.04.005
2. Kim KW, Ivanov S, Williams JW. Monocyte recruitment, specification, and function in atherosclerosis. *Cells*. 2020;10:15. doi: 10.3390/cells10010015
3. Mauersberger C, Hinterdobler J, Schunkert H, Kessler T, Sager HB. Where the action is-leukocyte recruitment in atherosclerosis. *Front Cardiovasc Med*. 2021;8:813984. doi: 10.3389/fcvm.2021.813984
4. Barrett TJ. Macrophages in atherosclerosis regression. *Arterioscler Thromb Vasc Biol*. 2020;40:20–33. doi: 10.1161/ATVBAHA.119.312802
5. Tabas I, Bornfeldt KE. Macrophage phenotype and function in different stages of atherosclerosis. *Circ Res*. 2016;118:653–667. doi: 10.1161/CIRCRESAHA.115.306256
6. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007;7:678–689. doi: 10.1038/nri2156
7. Imhof BA, Aurrand-Lions M. Adhesion mechanisms regulating the migration of monocytes. *Nat Rev Immunol*. 2004;4:432–444. doi: 10.1038/nri1375
8. Makgoba MW, Sanders ME, Ginther Luce GE, Dustin ML, Springer TA, Clark EA, Mannoni P, Shaw S. ICAM-1 a ligand for LFA-1-dependent adhesion of B, T and myeloid cells. *Nature*. 1988;331:86–88. doi: 10.1038/331086a0
9. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. *Nature*. 2002;417:182–187. doi: 10.1038/417182a
10. Carlos T, Kovach N, Schwartz B, Rosa M, Newman B, Wayner E, Benjamin C, Osborn L, Lobb R, Harlan J. Human monocytes bind to two cytokine-induced adhesive ligands on cultured human endothelial cells: endothelial-leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1. *Blood*. 1991;77:2266–2271. doi: 10.1182/blood.V77.10.2266.2266
11. Chuluyan HE, Issekutz AC. VLA-4 integrin can mediate CD11/CD18-independent transendothelial migration of human monocytes. *J Clin Invest*. 1993;92:2768–2777. doi: 10.1172/JCI116895
12. Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Gutierrez-Ramos JC, Connelly PW, Milstone DS. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin Invest*. 2001;107:1255–1262. doi: 10.1172/JCI11871
13. Nageh MF, Sandberg ET, Marotti KR, Lin AH, Melchior EP, Bullard DC, Beaudet AL. Deficiency of inflammatory cell adhesion molecules protects against atherosclerosis in mice. *Arterioscler Thromb Vasc Biol*. 1997;17:1517–1520. doi: 10.1161/01.atv.17.8.1517
14. Bourdillon MC, Poston RN, Covacho C, Chignier E, Bricca G, McGregor JL. ICAM-1 deficiency reduces atherosclerotic lesions in double-knockout mice (ApoE(-/-)/ICAM-1(-/-)) fed a fat or a chow diet. *Arterioscler Thromb Vasc Biol*. 2000;20:2630–2635. doi: 10.1161/01.atv.20.12.2630
15. Schneider JG, Zhu Y, Coleman T, Semenkovich CF. Macrophage beta3 integrin suppresses hyperlipidemia-induced inflammation by modulating TNFalpha expression. *Arterioscler Thromb Vasc Biol*. 2007;27:2699–2706. doi: 10.1161/ATVBAHA.107.153650
16. Basatemur GL, Jorgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. *Nat Rev Cardiol*. 2019;16:727–744. doi: 10.1038/s41569-019-0227-9
17. Bennett MR, Sinha S, Owens GK. Vascular smooth muscle cells in atherosclerosis. *Circ Res*. 2016;118:692–702. doi: 10.1161/CIRCRESAHA.115.306361
18. Misra A, Feng Z, Chandran RR, Kabir I, Rotllan N, Aryal B, Sheikh AQ, Ding L, Qin L, Fernandez-Hernando C, et al. Integrin beta3 regulates clonality and fate of smooth muscle-derived atherosclerotic plaque cells. *Nat Commun*. 2018;9:2073. doi: 10.1038/s41467-018-04447-7
19. Debrand E, El Jai Y, Spence L, Bate N, Praekelt U, Pritchard CA, Monkley SJ, Critchley DR. Talin 2 is a large and complex gene encoding multiple transcripts and protein isoforms. *FEBS J*. 2009;276:1610–1628. doi: 10.1111/j.1742-4658.2009.06893.x
20. von Essen M, Rahikainen R, Oksala N, Raitoharju E, Seppala I, Mennander A, Sioris T, Kholova I, Klopp N, Illig T, et al. Talin and vinculin are downregulated in atherosclerotic plaque; tampere vascular study. *Atherosclerosis*. 2016;255:43–53. doi: 10.1016/j.atherosclerosis.2016.10.031
21. Wei X, Sun Y, Wu Y, Zhu J, Gao B, Yan H, Zhao Z, Zhou J, Jing Z. Down-regulation of Talin-1 expression associates with increased proliferation and migration of vascular smooth muscle cells in aortic dissection. *BMC Cardiovasc Disord*. 2017;17:162. doi: 10.1186/s12872-017-0588-0
22. Lagarrigue F, Kim C, Ginsberg MH. The Rap1-RIAM-talin axis of integrin activation and blood cell function. *Blood*. 2016;128:479–487. doi: 10.1182/blood-2015-12-638700
23. Kim M, Carman CV, Springer TA. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science*. 2003;301:1720–1725. doi: 10.1126/science.1084174
24. Yago T, Petrich BG, Zhang N, Liu Z, Shao B, Ginsberg MH, McEver RP. Blocking neutrophil integrin activation prevents ischemia-reperfusion injury. *J Exp Med*. 2015;212:1267–1281. doi: 10.1084/jem.20142358
25. Garcia-Bernal D, Parmo-Cabanas M, Dios-Esponera A, Samaniego R, Hernán-P de la Ossa D, Teixido J. Chemokine-induced Zap70 kinase-mediated dissociation of the Vav1-talin complex activates alpha4beta1 integrin for T cell adhesion. *Immunity*. 2009;31:953–964. doi: 10.1016/j.immuni.2009.09.021
26. Hyduk SJ, Rullo J, Cano AP, Xiao H, Chen M, Moser M, Cybulsky MI. Talin-1 and kindlin-3 regulate alpha4beta1 integrin-mediated adhesion stabilization, but not G protein-coupled receptor-induced affinity upregulation. *J Immunol*. 2011;187:4360–4368. doi: 10.4049/jimmunol.1003725
27. Manevich-Mendelson E, Grabovsky V, Feigelson SW, Cinamon G, Gore Y, Goverse G, Monkley SJ, Margalit R, Melamed D, Mebius RE, et al. Talin1 is required for integrin-dependent B lymphocyte homing to lymph nodes and the bone marrow but not for follicular B-cell maturation in the spleen. *Blood*. 2010;116:5907–5918. doi: 10.1182/blood-2010-06-293506
28. Petrich BG, Marchese P, Ruggeri ZM, Spiess S, Weichert RA, Ye F, Tiedt R, Skoda RC, Monkley SJ, Critchley DR, et al. Talin is required for integrin-mediated platelet function in hemostasis and thrombosis. *J Exp Med*. 2007;204:3103–3111. doi: 10.1084/jem.20071800
29. Siegelman MH, Stanescu D, Estess P. The CD44-initiated pathway of T-cell extravasation uses VLA-4 but not LFA-1 for firm adhesion. *J Clin Invest*. 2000;105:683–691. doi: 10.1172/JCI8692
30. Shao B, Yago T, Panicker SR, Zhang N, Liu Z, McEver RP. Th1 cells rolling on selectins trigger DAP12-dependent signals that activate integrin alphaLbeta2. *J Immunol*. 2020;204:37–48. doi: 10.4049/jimmunol.1900680
31. Daugherty A, Tall AR, Daemen M, Falk E, Fisher EA, Garcia-Cardena G, Lusis AJ, Owens AP 3rd, Rosenfeld ME, Virmani R, et al. Recommendation on design, execution, and reporting of animal atherosclerosis studies: a scientific statement from the American Heart Association. *Arterioscler Thromb Vasc Biol*. 2017;37:e131–e157. doi: 10.1161/ATV.0000000000000062
32. Shao B, Yago T, Coghill PA, Klopocki AG, Mehta-D'souza P, Schmidtke DW, Rodgers W, McEver RP. Signal-dependent slow leukocyte rolling does not require cytoskeletal anchorage of P-selectin glycoprotein ligand-1 (PSGL-1) or integrin alphaLbeta2. *J Biol Chem*. 2012;287:19585–19598. doi: 10.1074/jbc.M112.361519
33. Hyduk SJ, Chan JR, Duffy ST, Chen M, Peterson MD, Waddell TK, Digby GC, Szaszi K, Kapus A, Cybulsky MI. Phospholipase C, calcium, and calmodulin are critical for alpha4beta1 integrin affinity up-regulation and monocyte arrest triggered by chemoattractants. *Blood*. 2007;109:176–184. doi: 10.1182/blood-2006-01-029199
34. Panicker SR, Yago T, Shao B, McEver RP. Neutrophils lacking ERM proteins polarize and crawl directionally but have decreased adhesion strength. *Blood Adv*. 2020;4:3559–3571. doi: 10.1182/bloodadvances.2020002423
35. Shattil SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. *Nat Rev Mol Cell Biol*. 2010;11:288–300. doi: 10.1038/nrm2871
36. Finney AC, Stokes KY, Pattillo CB, Orr AW. Integrin signaling in atherosclerosis. *Cell Mol Life Sci*. 2017;74:2263–2282. doi: 10.1007/s00018-017-2490-4
37. Eriksson EE. Intravital microscopy on atherosclerosis in apolipoprotein E-deficient mice establishes microvessels as major entry pathways for leukocytes to advanced lesions. *Circulation*. 2011;124:2129–2138. doi: 10.1161/CIRCULATIONAHA.111.030627
38. Chigaev A, Blenc AM, Braaten JV, Kumaraswamy N, Kepley CL, Andrews RP, Oliver JM, Edwards BS, Prossnitz ER, Larson RS, et al. Real

time analysis of the affinity regulation of alpha 4-integrin. The physiologically activated receptor is intermediate in affinity between resting and Mn(2+) or antibody activation. *J Biol Chem*. 2001;276:48670–48678. doi: 10.1074/jbc.M103194200
39. Arnaout MA, Goodman SL, Xiong JP. Coming to grips with integrin binding to ligands. *Curr Opin Cell Biol*. 2002;14:641–651. doi: 10.1016/s0955-0674(02)00371-x
40. Nieswandt B, Varga-Szabo D, Elvers M. Integrins in platelet activation. *J Thromb Haemost*. 2009;7:206–209. doi: 10.1111/j.1538-7836.2009.03370.x
41. Ripamonti M, Wehrle-Haller B, de Curtis I, de Curtis I. Paxillin: a hub for mechano-transduction from the beta3 integrin-talin-kindlin axis. *Front Cell Dev Biol*. 2022;10:852016. doi: 10.3389/fcell.2022.852016
42. Mestas J, Ley K. Monocyte-endothelial cell interactions in the development of atherosclerosis. *Trends Cardiovasc Med*. 2008;18:228–232. doi: 10.1016/j.tcm.2008.11.004
43. Lu X, Lu D, Scully MF, Kakkar VV. The role of integrin-mediated cell adhesion in atherosclerosis: pathophysiology and clinical opportunities. *Curr Pharm Des*. 2008;14:2140–2158. doi: 10.2174/138161208785740199
44. Tedder TF, Steeber DA, Pizcueta P. L-selectin-deficient mice have impaired leukocyte recruitment into inflammatory sites. *J Exp Med*. 1995;181:2259–2264. doi: 10.1084/jem.181.6.2259
45. Shi C, Velazquez P, Hohl TM, Leiner I, Dustin ML, Pamer EG. Monocyte trafficking to hepatic sites of bacterial infection is chemokine independent and directed by focal intercellular adhesion molecule-1 expression. *J Immunol*. 2010;184:6266–6274. doi: 10.4049/jimmunol.0904160
46. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. 2003;19:71–82. doi: 10.1016/s1074-7613(03)00174-2
47. Galkina E, Ley K. Vascular adhesion molecules in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2007;27:2292–2301. doi: 10.1161/ATVBAHA.107.149179
48. Woodfin A, Voisin MB, Beyrau M, Colom B, Caille D, Diapouli FM, Nash GB, Chavakis T, Albelda SM, Rainger GE, et al. The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. *Nat Immunol*. 2011;12:761–769. doi: 10.1038/ni.2062
49. Llodra J, Angeli V, Liu J, Trogan E, Fisher EA, Randolph GJ. Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proc Natl Acad Sci USA*. 2004;101:11779–11784. doi: 10.1073/pnas.0403259101
50. Feig JE, Quick JS, Fisher EA. The role of a murine transplantation model of atherosclerosis regression in drug discovery. *Curr Opin Investig Drugs*. 2009;10:232–238.
51. Sun Z, Costell M, Fassler R. Integrin activation by talin, kindlin and mechanical forces. *Nat Cell Biol*. 2019;21:25–31. doi: 10.1038/s41556-018-0234-9
52. Manevich E, Grabovsky V, Feigelson SW, Alon R. Talin 1 and paxillin facilitate distinct steps in rapid VLA-4-mediated adhesion strengthening to vascular cell adhesion molecule 1. *J Biol Chem*. 2007;282:25338–25348. doi: 10.1074/jbc.M700089200
53. Alon R, Kassner PD, Carr MW, Finger EB, Hemler ME, Springer TA. The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. *J Cell Biol*. 1995;128:1243–1253. doi: 10.1083/jcb.128.6.1243
54. Chen PY, Qin L, Li G, Tellides G, Simons M. Smooth muscle FGF/TGF-beta cross talk regulates atherosclerosis progression. *EMBO Mol Med*. 2016;8:712–728. doi: 10.15252/emmm.201506181
55. Zhao D, Li J, Xue C, Feng K, Liu L, Zeng P, Wang X, Chen Y, Li L, Zhang Z, et al. TL1A inhibits atherosclerosis in apoE-deficient mice by regulating the phenotype of vascular smooth muscle cells. *J Biol Chem*. 2020;295:16314–16327. doi: 10.1074/jbc.RA120.015486
56. Wei X, Sun Y, Han T, Zhu J, Xie Y, Wang S, Wu Y, Fan Y, Sun X, Zhou J, et al. Upregulation of miR-330-5p is associated with carotid plaque's stability by targeting Talin-1 in symptomatic carotid stenosis patients. *BMC Cardiovasc Disord*. 2019;19:149. doi: 10.1186/s12872-019-1120-5